Exhibit A

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A Gene Encoding an RNase D Exonuclease-Like Protein is Required for Posttranscriptional Silencing in *Arabidopsis*

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Summary

Posttranscriptional gene silencing (PTGS) and the closely related phenomenon, RNA interference (RNAi), result from the initial endonucleolytic cleavage of target mRNAs, which are then presumed to be completely hydrolyzed by exoribonucleases. To date, no plant genes required for PTGS are known to encode exoribonucleases. The *Arabidopsis Werner Syndrome-like exonuclease (WEX)* gene encodes an RNase D domain most similar to that in human Werner Syndrome protein (WRN), but lacks the RecQ helicase domain. It is also related to *Caenorhabditis elegans mut-7*, which is essential for RNAi, PTGS, and transposon activity. We isolated a loss-of-function mutant, *wex-1,* that showed greatly reduced expression of *WEX* mRNA and early flowering. Although *wex-1* did not affect expression of a robust marker for transcriptional gene silencing (TGS), PTGS of a green-fluorescent-protein (GFP) reporter gene was blocked in *wex-1* and restored by ectopic expression of WEX indicating that WEX is required for PTGS but not TGS. Thus, members of the RNase D protein family are required for PTGS in both plants and animals. Interestingly, WEX has been shown to interact with an *Arabidopsis* RecQ helicase suggesting these proteins might comprise a functional equivalent of WRN.

Introduction

Posttranscriptional gene silencing (PTGS) and the closely related phenomenon, RNA interference (RNAi), are epigenetic forms of mRNA degradation that occur in diverse eukaryotes (Tijsterman et al., 2002).

Although genetic and biochemical studies suggest that at least some components of PTGS/RNAi have been highly conserved in evolution, features such as transitivity, systemic spread, and downstream steps in RNA degradation can differ in different organisms (Tijsterman et al., 2002; Sijen et al., 2001; Vaistij et al., 2002). In addition, there is growing evdence for links between PTGS/RNAi and transcriptional gene silencing (TGS), which is accompanied by methylation of cytosines in the promoter region of the target genes (Vaucheret et al., 2001) (Aufsatz et al., 2002). At present, it is not clear if multiple mechanisms for PTGS exist and to what extent these mechanisms and those for TGS overlap at the molecular level.

PTGS in plants, like RNAi in animals, is probably initiated by double-stranded RNAs (dsRNA) that are processed by Dicer-like RNase III activity to small interfering RNA (siRNA) duplexes, which guide the endonucleolytic cleavage of cognate RNAs in the region of complementarity (Matzke et al., 2001b; Plasterk, 2002; Vaucheret et al., 2001; Tang et al., 2003). The resultant fragments are then completely hydrolyzed, presumably by exoribonucleases. Previous genetic and molecular studies of *Arabidopsis* have identified four genes necessary for PTGS. The *SGS2/SDE1* gene encoding a putative RNA-dependent RNA polymerase (RdRP), which may help synthesize dsRNAs (Dalmay et al., 2000; Mourrain et al., 2000; Vaistij et al., 2002); the *SDE3* gene encoding an RNA helicase (Dalmay et al., 2001); the *AGO1* encoding a PAZ-domain protein (Cerutti et al., 2000; Fagard et al., 2000); and, the *SGS3* gene encoding a plant-specific protein of unknown function with coiled-coil domains (Mourrain et al., 2000). (Mourrain et al., 2000). In addition, the *SIN1/SUS1/CAF* (DCL1) gene, which encodes a Dicer-

like protein (Schauer et al., 2002), has been shown to be important for the formation of micro RNAs from dsRNA (Reinhard et al., 2002; Golden et al., 2002)To date, no genes encoding exoribonucleases have been implicated in plant PTGS.

The *C. elegans mut-7* gene has been shown to be essential for RNAi, PTGS, and silencing of transposon activity (Tabara et al., 1999; Ketting et al., 1999). It encodes a member of the RNase D protein family belonging to the DEDD superfamily of 3'-5' exoribonucleases (Zuo and Deutscher, 2001; Mian, 1997). Some members of this family are important for processing and maturation of small RNA species such as tRNAs and snRNAs in *E.coli* and yeast (Zuo and Deutscher, 2001). Thus, MUT-7 may function in the degradation of mRNAs targeted by PTGS or RNAi. The MUT-7 protein is similar to the human Werner syndrome protein (WRN) (Yu et al., 1996), which has been implicated in premature aging and contains functional RecQ helicase and RNase D domains (Shen et al., 1998; Shen and Loeb, 2000).

Here we show that the *Arabidopsis Werner Syndrome-like exonuclease (WEX)* gene, which encodes an RNase D protein related to WRN and MUT-7 is required for PTGS, but not for TGS.

Results

Identification of Arabidopsis RNase D family members

The *C. elegans* MUT-7 protein is the only member of the RNase D family known to be implicated in PTGS. We used BLASTp (Altschul et al., 1990) and Hidden Markov Model searches (Eddy, 1996) done with the 3'-5' exonuclease Pfam profile (01612) (Bateman et al., 2000) to find *Arabidopsis* homologues with a similar role in plant PTGS. We identified six proteins with potential RNase D domains (HMM E-values < $1e^{-10}$): AAD25623, AAC69936, CAB36851, AAC42241, AAG50917 and BAB11227 (Supplemental Table 2). These proteins show sequence conservation in the three Exo motifs that cluster around the active site of the RNase D domain with the five most conserved residues present in all the proteins, except for the tyrosine in AAC42241 (Mian, 1997)CAB36851 was most closely related to MUT-7 (BLASTp E-value = $7e^{-4}$) and had an RNase D-like domain most similar to that in human WRN. We call the gene encoding CAB36851, previously designated *AtWRNexo* (Hartung et al., 2000), the *Werner Syndrome-like exonuclease (WEX)* to comply with the accepted nomenclature for *Arabidopsis* genes.

We isolated a *WEX* cDNA (AF531179) by RACE and found that the WEX protein differs from that predicted in CAB36851. All twelve 3' RACE RT-PCR products examined for this cDNA have an additional nine bases (869 to 877) not present in the published cDNA (AJ404476) (Hartung et al., 2000), suggestive of alternative splicing. A closely related rice gene produces two transcripts differing by nine bp due to alternative 3' splice sites at the same position in the protein (J.Z.L., unpublished data). This portion of the WEX protein is predicted to be part of an alpha helix in the Exo III motif close to the C-terminus of the protein (Mian, 1997). Examination of EST sequences in

GenBank provides evidence for what appears to be an expressed pseudogene with sequence similarity to *WEX* upstream of the *WEX* gene (see supplemental Figure 6).

The T-DNA insertion mutant wex-1 shows greatly reduced WEX mRNA expression

We obtained the loss-of-function *wex-1* mutant by PCR screening of a large pool of T-DNA lines (Krysan et al., 1999). This mutant contains a T-DNA insertion in the 5'-UTR 26 bp upstream of the start codon. Figure 1 shows that *WEX* mRNA expression of *wex-1* plants was greatly reduced relative to wild type. *WEX* mRNA could be detected by RNA-blot hybridization of poly (A^+) RNA from wild type plants but not *wex-1* plants (Fig. 1A). The *WEX* RT-PCR product was readily detectable with total RNA from wild-type plants, but only barely detectable with total RNA from the *wex-1* mutant, even when 10-fold more of the reaction mixture was loaded on the gel (Fig. 1B).

Although *wex-1* plants showed greatly reduced levels of *WEX* mRNA, they did not show visible abnormalities and were morphologically indistinguishable from wild type at the embryo, seedling, and flowering stages (Fig. 2A,E and data not shown). Interestingly, preliminary data suggests that under our standard conditions (16h light/ 8h darkness, 21° C, 65% humidity), *wex-1* plants flowered 10-15 days earlier than wild type, which flowered after 40-50 days (Fig. 2 A,B).

Characterization of a PTGS indicator line

To assay for effects of *wex-1* on PTGS, we generated the silent indicator line 8z2. This line and the high-GFP expressing line 2, which served as a positive control, are independent, monogenic lines homozygous for a chimeric *GFP* reporter gene regulated by a duplicated cauliflower mosaic virus 35S RNA promoter ($35S_2$-GFP). We partially characterized the T-DNA inserts by Southern-blot hybridization (supplemental Figure 7) and mapped their positions in the *Arabidopsis* genome by TAIL-PCR. Line 8z2 contains a T-DNA direct tandem repeat inserted at position 42963 in genomic clone F22L4 on chromosome 1 (AC061957), which was confirmed by sequencing the entire locus. Line 2 contains a single T-DNA insert with its left border at position 46355 in genomic clone T16F16 (AC005312) on chromosome 2.

Control line 2 plants showed uniform, high-level GFP expression at rosette stage (Fig. 2C) and throughout sporophytic development in more than 500 plants scored (data not shown). Line 8z2 plants exhibited resetting of silencing, which is characteristic of PTGS (Meins, Jr., 2000), i.e., cotyledon-stage seedling were high-GFP expressing. Silencing started at the two-leaf stage, which was evident in essentially all visible parts of the plants by mid-rosette stage (Fig. 2D) and then persisted throughout sporophytic development (data not shown). Of more than 500 plants scored in several experiments, the incidence of silencing at mid-rosette stage ranged from 90-100%. No silencing was detected in plants hemizygous for the *GFP* transgene (data not shown). RNA-blot hybridization confirmed a marked reduction of *GFP* mRNA in silent line 8z2 plants relative to that of high-GFP expressing

line 2 plants (Fig. 3 A). Comparison of these steady-state mRNA levels with those of nascent *GFP* RNA determined by nuclear run-on transcription measurements (Fig. 3 B), showed that silencing of GFP expression in line 8z2 results from PTGS rather than from a decreased rate of transcription. This conclusion was confirmed by the finding that small, ca. 21-25 nt long RNAs (smRNAs) considered diagnostic for PTGS (Hamilton and Baulcombe, 1999) accumulated in silent line 8z2 tissues, but not in high- GFP expressing line 2 tissues (Fig. 4).

WEX Expression is Required for PTGS, but not for TGS

We obtained plants homozygous for *wex-1* and for the *35S$_2$-GFP* T-DNA by crossing the *wex-1* mutant with line 8z2 and PCR genotyping the F_2 generation with gene-specific primers that span the insertion sites. Populations of *wex-1/wex-1* and *WEX/WEX* plants in the F3 generation and parental 8z2 plants were scored for incidence of PTGS. Measurements of *WEX* mRNA confirmed that neither the presence of the GFP transgene nor silencing of this gene had appreciable effects on expression of the wild type *WEX* and *wex-1* alleles (Fig. 1A,B).

The *wex-1* mutation strongly inhibited GFP PTGS. Plants homozygous for both the 35S$_2$-GFP locus and *wex-1*, which had greatly reduced *WEX* mRNA contents relative to untransformed *WEX* plants (Fig. 1A,B), showed high-levels of GFP fluorescence (Fig. 2C,E) and *GFP* mRNA accumulation (Fig. 4 A) comparable to the line 2 control, and, like the high-GFP expressing

line 2, did not accumulate *GFP* smRNAs (Fig. 4 B). None of the 36 plants scored exhibited PTGS, which is significantly different from the high incidence found with the parental 8z2 line or the outcrossed line carrying the wild-type *WEX* allele (Table 1).

Complementation experiments confirmed that *WEX* expression is required for PTGS. We selected monogenic, homozygous Ubq3-WEX transformants carrying the *WEX* cDNA and a hygromycin-resistance marker that expressed appreciably higher *WEX* mRNA levels than wild type (data not shown). These lines were crossed with line T15 (*wex-1/wex-1* $35S_2$-*GFP/*$35S_2$-*GFP*; Table 1) and the F2 generation was screened for hygromycin-resistant *wex-1/wex-1* $35S_2$-*GFP/*$35S_2$-*GFP* plants. PTGS was restored in *wex-1/wex-1* plants carrying at least one copy of the Ubq3-WEX transgene. The incidence of GFP silencing in the complemented line TW, 92%, was significantly higher than in the *wex-1/wex-1* line, but did not differ significantly from the incidence of silencing in *WEX/WEX* lines (Table 1).

To examine possible effects of the *wex-1* mutation on TGS, we measured expression of transcriptionally silent information (TSI) RNAs, which have been shown to be robust markers for TGS (Steimer et al., 2000). These RNAs are expressed in several TGS-defective mutants, including *mom1*, but not in wild type. RNA-blot hybridization showed that the two RNAs detected with a probe for TSI-B RNA are expressed in *mom1,* but not in wild-type or *wex-1* plants (data not shown). Thus, WEX does not appear to be required for TGS.

Discussion

RNase D protein family members are required for PTGS in both plants and animals

Our results together with recent studies of the *C. elegans mut-7* mutant (Tabara et al., 1999) show that members of the RNase D protein family are required for PTGS in both plants and animals. *wex-1* plants show greatly reduced accumulation of WEX mRNA and are deficient in PTGS, which is restored by ectopic expression of a *WEX* transgene. This strongly suggests that *wex-1* is a recessive, loss-of-function mutant and that the *WEX* gene product is required for PTGS. The *C. elegans mut-7* mutant shows heritable parent-of-origin effects, which are often associated with TGS in other organisms (Reik et al., 2001), and frequently loses its X chromosome during meiosis (Ketting et al., 1999). Mutations in the human *WRN* gene lead to similar genetic instabilities and premature onset of age-related diseases (Shen and Loeb, 2000). By contrast, the *wex-1* mutant did not show obvious effects on the growth or development of *Arabidopsis* other than early flowering. Although decreased DNA methylation can result in early flowering under short day conditions (Sheldon et al., 1999), TGS was not blocked in *wex-1*.

Interacting WEX and RecQ helicases in Arabidopsis could be the functional equivalent of WRN

The domain structures of several WEX-related proteins are shown in Figure 5. *Arabidopsis* WEX and *C. elegans* MUT-7 are closely related to human WRN in the RNase D family, but lack a RecQ helicase domain. Interestingly, the *Neurospora* QDE3 protein, which is required for PTGS, has a RecQ helicase domain, but no RNase D domain (Cogoni and Macino, 1999). Six members of the RecQ helicase family have been identified in *Arabidopsis* (Hartung et al., 2000). One of these, AtRecQl2, has been shown to interact with WEX in a yeast two-hybrid system. Together these findings lead us to speculate that in *Arabidopsis*, an interacting RecQ helicase and RNase D domain protein could be the functional equivalent of WRN. It is not known if both domains are required for PTGS.

Where does WEX act in PTGS pathway?

WEX is a member of the DEDD superfamily of 3'-5' exoribonucleases, which includes proteins important for RNA processing and (Zuo and Deutscher, 2001; Mian, 1997) (van Hoof and Parker, 1999). WEX, however, does not have an essential housekeeping function since the *wex-1* mutant is viable and develops normally. WEX appears to have PTGS-specific functions. Where and how WEX acts in the PTGS pathway is not known. Considerable evidence suggests that plant siRNAs guide the degradation of cognate RNAs in a RISC-like endonuclease complex (Tang et al., 2003). This involves an initial endonucleolytic cleavage and subsequent hydrolysis of target RNAs, presumably mediated by 3' 5'- and 5' 3'-exoribonucleases (Meins, 2000; Matzke et al., 2001a; Matzke et al., 2001b). Thus, WEX could specifically degrade the products of endonuclease activity. This hypothesis is difficult to

reconcile with our finding that WEX is required for PTGS, since the initial endonucleolytic cleavage of the RNA should be sufficient to impair its translational activity. Nevertheless, *GFP* mRNA levels as well as GFP expression were restored in the *wex-1* mutant; and, no truncated *GFP* mRNAs likely to form if degradation were blocked after initial endonucleolytic cleavage were detected (data not shown). Two possible explanations are that WEX is an essential component of the RISC-like endonuclease complex or that the removal of endonuclease products from the complex is required for siRNA-guided RNA degradation. Another possibility, which we favor, is that WEX acts upstream of RISC. This working hypothesis is consistent with our finding that smRNAs, presumably generated by Dicer-like activity, accumulate in silent WEX/WEXGFP plants, but not in wex-*1/wex-1* GFP plants impaired in *GFP* RNA silencing. Finally, we cannot rule out the possibility that WEX acts at more than one site, e.g., upstream and downstream of siRNA formation, as has been proposed for putative RNA dependent RNA polymerases(Dalmay et al., 2000; Mourrain et al., 2000; Vaistij et al., 2002; Klahre et al., 2002).

Experimental procedures

Isolation of a WEX cDNA

Primers for 5' and 3' rapid amplification of cDNA ends (RACE) were designed based on the exon/intron boundaries for the predicted protein (CAB36851). PCR products from 5' and 3' RACE (GeneRacer kit, Invitrogen) were TA-

cloned (Original TA-Cloning kit, Invitrogen) and the longest clones were assembled to form an 1150 bp *WEX* cDNA sequence (AF531179).

Isolation of the wex-1 mutant

A pool of ~60,480 independent T-DNA lines of *Arabidopsis* ecotype Wassilewskija (Krysan et al., 1999) was screened by PCR utilizing primers corresponding to the pD991 T-DNA left border and the *WEX* 3'-specific region. These lines carry an *nptII* gene, which confers kanamycin resistance, regulated by the *mas* promoter and transcriptional terminator. De-convolution of pool architecture and genotyping by PCR led to the identification a line homozygous for the insertion, which was designated *wex-1*.

Plant material

Unless indicated otherwise *Arabidopsis thaliana* ecotype Columbia plants were used. Transgenic plants were obtained by Agrobacterium-mediated transformation (Bechtold N. and Pelletier G., 1998) with two Ti-plasmids. p35S_2-GFP carries a chimeric GFP (Reichel et al., 1996) reporter gene regulated by a duplicated cauliflower mosaic virus 35S RNA promoter and transcriptional terminator (Goodall and Filipowicz, 1989) and an *nptII* gene regulated by the *nos* promoter and transcriptional terminator in binary vector pBIN19 (Bevan, 1984). pUbq3-WEX carries the coding region of *WEX* cDNA regulated by the *Arabidopsis ubiquitin 3* promoter (Norris et al., 1993) and *nos* transcriptional terminator and an *hptII* gene, which confers hygromycin resistance, regulated by a duplicated cauliflower mosaic virus 35S RNA

promoter and transcriptional terminator in binary vector pCAMBIA-1380 (CAMBIA, Canberra, Australia). Monogenic transformants homozygous for the resistance markers were obtained from the F2 generation of selfed transformants.

Assaying for GFP fluorescence

GFP fluorescence was monitored by illuminating plants with UV light using a "BLAK-RAY®" semiconductor inspection lamp (Omnilab).

DNA Analysis

Total genomic DNA was extracted from leaf tissue using the Nucleon PhytoPure DNA extraction kit (Amersham Life Science). Southern blot hybridization was performed with digoxigenin (DIG)-labeled DNA probes synthesized using a PCR DIG Probe Synthesis Kit (Roche). Primers for amplification of *GFP* and *nptII* probes were GFP1 (5'-AAGGAAGAGGAGCTCTTCACCG), GFP2 (5'-TTCTGCTGGTAGTGGTCGGC), NPTII1 (5'-CGCATGATTGAA CAAGATGG), and NPTII2 (5'-AACGATTCCGAAGCCCAAC).

The genomic position of the T-DNAs was determined by thermal asymmetric interlaced PCR (TAIL-PCR) (Liu et al., 1995) using T-DNA specific primers LB1 (5'-TTCGGAACCACCATCAAACAGG) LB2 (5'-TTGCTGCAACTCTCTCAGGGCC), LB3 (5'-TCAGCTGTTGCCCGTCTCACT) and degenerate primer AD3 (5'-WGTGNAGWANCANAGA, where W=A/T and N=G/A/T/C). Amplified PCR fragments were ligated in to pGEM-T Easy vector

(Promega), sequenced, and used as a query in BLAST searches to identify genomic DNA adjacent to a T-DNA border. Transformants were genotyped using the T-DNA specific primer 5'-CATTTTATAATAACGCTGCGGACATCTAC and *WEX* gene specific primer 5'-CGACATGATCTGATACATCGTTATGCCAAT to detect the insertion in *WEX*; the T-DNA specific primer LB1 and gene specific primers 5'-TTCGAAAACATTACCTCCGATC and 5'-GGCTTTTGCATTTGGTATCTACTAG were used to detect the $35S_2$-GFP T-DNA insertion.

RNA Analysis

Total RNA was extracted from leaf tissue using TRIZOL reagent (Gibco BRL). Poly (A^+) RNA was isolated using a Poly (A) Pure mRNA purification kit (Ambion). High-molecular weight and low-molecular weight RNA fractions of the same total RNA preparation were obtained using a RNeasy Midi Kit (Qiagen) as modified by Di Serio et a.l (Di Serio et al., 2001). For RNA-blot hybridization, 10 µg aliquots of RNA were loaded onto a 0.8% (w/v) denaturing agarose gel containing 50% (v/v) of formaldehyde. RNA blotting and hybridization of high-molecular weight RNAs was done using DIG-labeled DNA probes (Roche). The hybridization probes used to detect sense *GFP* smRNAs were a mixture of ^{32}P end-labeled oligodeoxynucleotides covering the *GFP* coding region. TSI RNA was measured with a probe for TSI-B as described (Steimer et al., 2000). For reverse-transcription coupled with PCR amplification (RT-PCR), cDNA was synthesized using Omniscript Reverse

Transcriptase (Qiagen), utilizing 1 μg of total RNA as a template for the randomly-primed reverse transcription reaction. cDNAs obtained from the reverse transcription reaction were used for subsequent PCR amplification of *WEX*. The primers WEX CDS_F (5'-ATGTCATCGTCAAATTGGATCGACG) and WEX_R (5'-CGCTTATCAACCTCAGTAGCAGTCTTG) were designed to amplify a 329 bp fragment spanning a 5' region of the coding sequence. Nuclear run-on transcription experiments (van Blokland et al., 1994) were done using hybridization probes for GFP (Reichel et al., 1996) and the *Arabidopsis actin-1* gene (M20016).

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Table 1. Effect of the *wex-1* mutation on the incidence of *35S$_2$-GFP* PTGS

Line	Description	Incidence of PTGS (%)*
8z2	P_1 Generation *WEX/WEX 35S$_2$-GFP/35S$_2$-GFP*	90 (40)[a]
T3	F_3 Generation *WEX/WEX 35S$_2$-GFP/35S$_2$-GFP*	88 (33)[b]
T15	F_3 Generation *wex-1/wex-1 35S$_2$-GFP/35S$_2$-GFP*	0 (36)[a,b,c]
TW	*Complemented T15* *wex-1/wex-1 35S$_2$-GFP/35S$_2$-GFP + Ubq3-WEX cDNA*	92 (27)[c]

* Percentage of the total number of plants scored shown in parenthesis exhibiting a silent phenotype at rosette stage. Values with the same letter are significantly different ($P<0.0005$, Binomial Proportions Test)

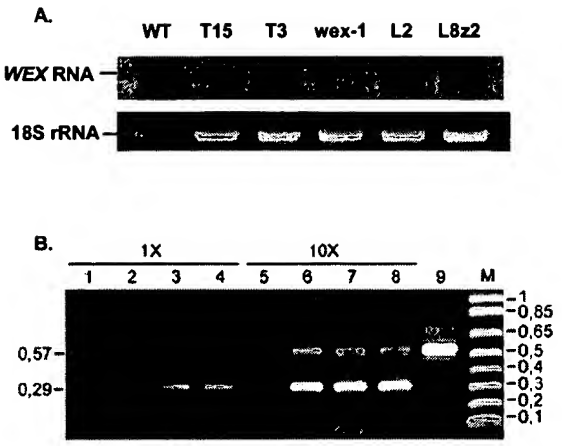


Figure 1. *wex-1* shows greatly reduced *WEX* mRNA expression.

(A) RNA-blot hybridization of poly (A^+) RNA prepared from wild type (WT), *wex-1/wex-1 35S$_2$-GFP /35S$_2$-GFP* (T15), *WEX/WEX 35S$_2$-GFP /35S$_2$-GFP* (T3), *wex-1/wex-1* (*wex-1*), line 2 (L2), and line 8z2 (L8z2) plants using *WEX* cDNA as a hybridization probe. The 18s rRNA used as a loading standard was stained with ethidium bromide.

(B) RT-PCR with cDNAs amplified for 30 PCR cycles. A 0.8 % agarose gel was loaded with 10 µl of the PCR reaction mixture (10x) or a 1/10 dilution of this mixture (1x) obtained with *wex-1* cDNA (lanes 1 and 5), wild-type cDNA (lanes 2 and 6), line 8z2 cDNA (lanes 3 and 7), line 2 cDNA (lanes 4 and 8), and wild-type genomic DNA (lane 9). Gels were stained with ethidium bromide. The sizes of the molecular weight markers (lane M) in kb are indicated on the right; the sizes of the amplification products on the left. The PCR product amplified using genomic DNA gave the expected 570 bp product expected for the *WEX* primers used.

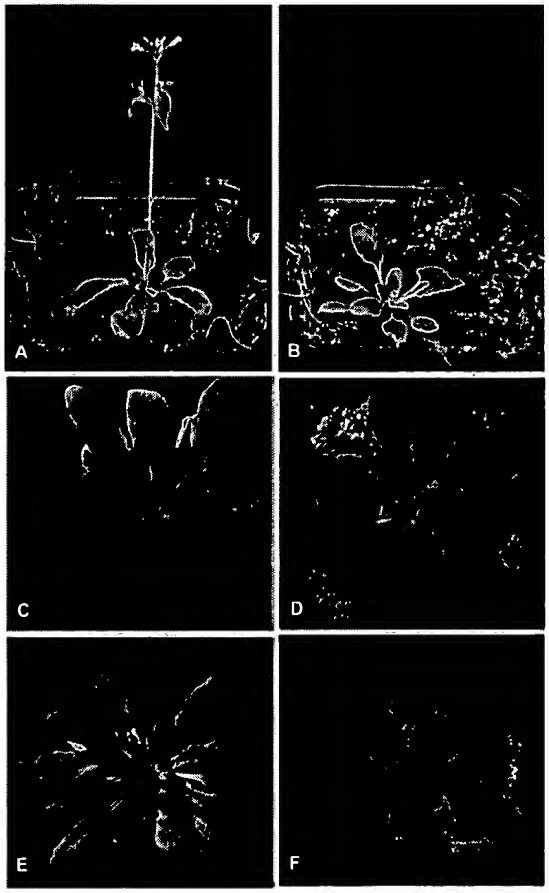


Figure 2. Expression of GFP in representative $35S_2$-GFP transformants.

(A-B) *wex-1* mutant (A) and wild type (B) plants illuminated with white light and photographed 7 days after germination.

(C-F) Representative line 2 plants, which show stable, high-level GFP expression (C), silent 8z2 plants (D), F_3 generation T15 *wex-1/wex-1* $35S_2$-GFP/ $35S_2$-GFP plants (E), and wild-type, untransformed plants photographed at rosette stage under UV illumination. Plants illuminated by UV light exhibit green fluorescence when GFP is expressed and red chlorophyll fluorescence when GFP expression is silenced.

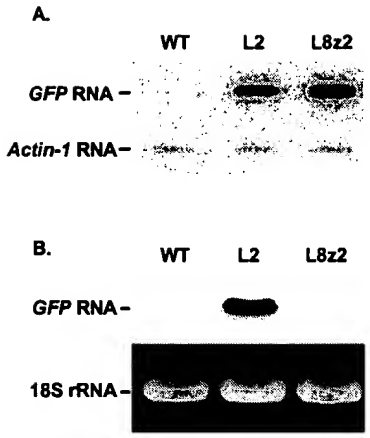


Figure 3. Silencing of *GFP* mRNA accumulation in line 8z2 is at the posttranscriptional level.

(A) Nuclear run-on transcription measurements of *GFP* RNA using *Arabidopsis actin-1* RNA as a control and nuclei prepared from wild-type, untransformed (WT), line 2 (L2), and line 8z2 (L8z2) leaves.

(B) RNA blot hybridization with a probe for *GFP* RNA of poly (A^+) RNA prepared from the same pooled tissues used in the nuclear run-on transcription experiment shown in (A).

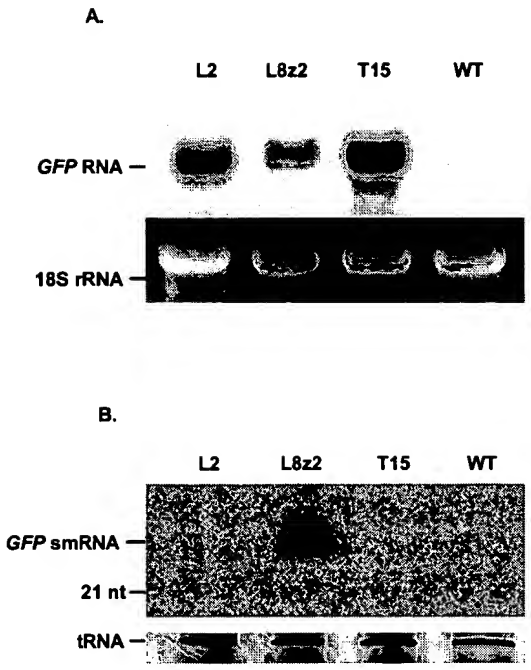


Figure 4. Accumulation of *GFP* smRNAs is associated with GFP PTGS

(A-B) RNA-blot hybridization of high- and low-molecular weight fractions of total RNA prepared from line 2 (L2), line 8z2 (L8z2), *wex-1/wex-1 35S$_2$-GFP /35S$_2$-GFP* (T15) and wild-type, untransformed (WT) leaf tissue. Loading standards, 18S rRNA and a selected tRNA as indicated, were stained with ethidium bromide. (A) High molecular weight fraction hybridized with a probe for *GFP* mRNA. (B) Low molecular weight fraction hybridized with a probe for sense *GFP* RNA. The position of the 21 nt oligoribonucleotide size standard is indicated.

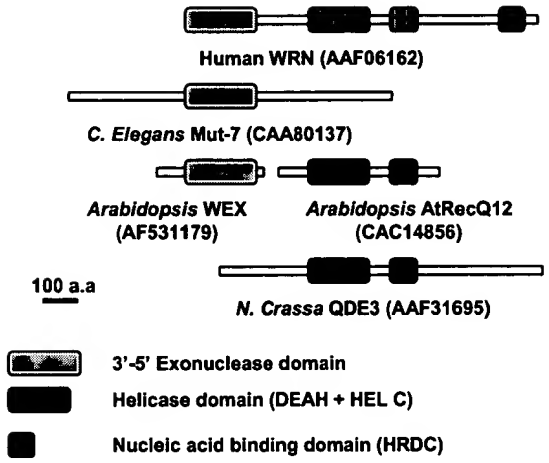


Figure 5. Schematic domain structure of WRN-like proteins.

Accession numbers are indicated in parentheses. Sizes of proteins and domains in amino acid residues are approximate. We speculate that two proteins in *Arabidopsis*, WEX and AtRecQl2, which are binding partners, could be the functional equivalent of WRN in humans.

Supplemental Data

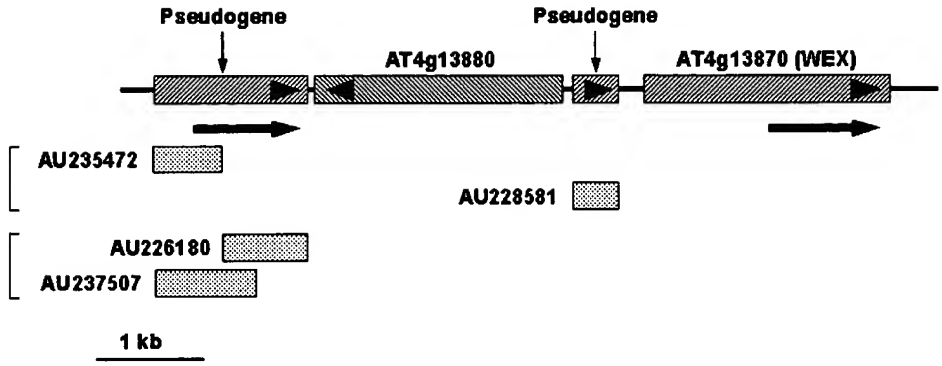
Supplemental Tabl 2. *Arabidopsis* RNase D domain proteins

Accession number	HMM E-value*	Position of conserved Exo motifs in RNase D domain proteins		
		Exo I	Exo II	Exo III
CAB36851 (WEX)†	$7.9e^{-15}$	D133, E135	D194	Y263,D 267
AAC69936	$2.3e^{-77}$	D80, E82	D138	Y203, D207
AAD25623	$4.3e^{-63}$	D140, E142	D199	Y264,D 268
AAC42241‡	$1.0e^{-23}$	D50, E52	D108	A192, D196
BAB11227	$1.5e^{-14}$	D34, E36	D104	Y186, D190
AAG50917	$6.5e^{-14}$	D384, E386	D449	Y531,D 535
CAA80137 (MUT-7)	$3.7e^{-62}$	D435,E437	D503	Y585, D589
AAF06162 (WRN)	$5.3e^{-56}$	D82, E84	D143	Y212, D216

* Values for 3'-5'-exonuclease profile PFAM PF01612

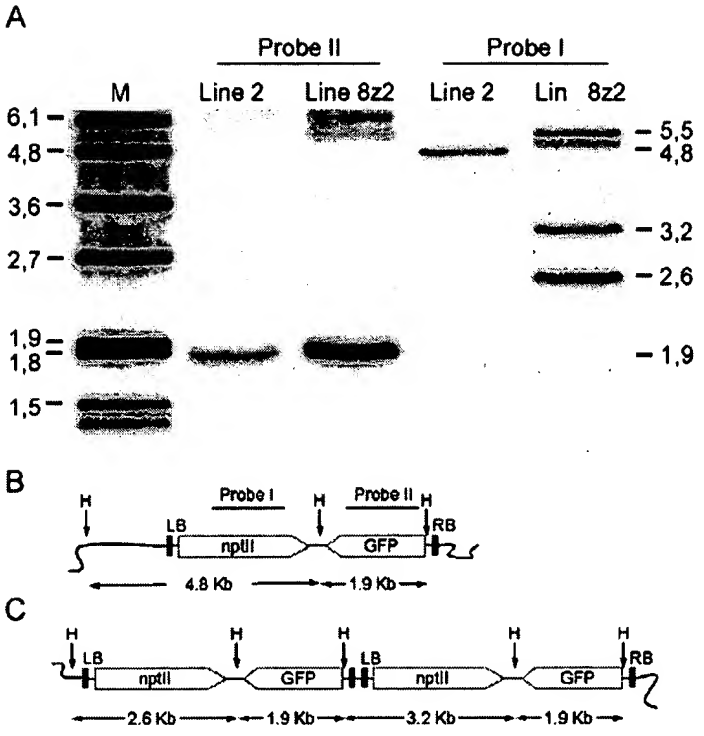
† A conceptual translation of the *WEX* cDNA sequence was used instead of the incorrect prediction in CAB36851

‡ Analysis done with a modified sequence that includes an additional 64 amino acids at the N-terminus



Supplemental Figure 6. ESTs for an expressed pseudogene in the *WEX* genomic region.

This pseudogene seems to have two transcripts differing at their 3' end. The shorter transcript was observed in RAFL14-14-A20 (AU235472, AU226180), which starts at 101101 and ends at 99695 on BAC F18A5. The longer transcript was observed in RAFL16-31-E24 (AU237507, AU228581), which starts at 101089, has an undefined section between the two ESTs, and ends at 96820. The *WEX* mRNA starts at 96598 and ends at 94336. Positions of genes are shown as shaded boxes on the top line. Arrowheads in these boxes indicate the direction of transcription. Brackets indicate ESTs derived from the same clone. The thick arrows indicate a region of very high sequence similarity shared by the pseudogene and *WEX*. Scale bar is 1 kb.



Supplemental Figure 7. Southern-blot analyses of lines 8z2 and 2

We partially characterized the T-DNA inserts in GFP-transformed lines 2 and 8z2 by Southern-blot hybridization using probes for the coding region of *GFP* and *nptII*. The T-DNA region of the expression vector contains two *Hind* III sites, one near the right border downstream of the *GFP* gene, and a second one located between the *nptII* and *GFP* genes. The Figure shows that *Hind* III-digested DNA prepared from line 2 gave the simple hybridization pattern expected for a single T-DNA insert (Panel B). *Hind* III-digested DNA prepared from line 8z2 showed a more complex hybridization pattern (Panel A). Based on the number and length of the fragments, we propose that this line has two full length T-DNA copies arranged in a direct tandem repeat (Panel C). This conclusion was confirmed by sequencing the entire 8z2 locus